

IN THE SPECIFICATION:

Please amend the paragraph beginning on page 4, line 1 as follows:

Trichoderma reesei. Preferably, the α -1,2-mannosidase expression vector is engineered such that the α -1,2-mannosidase or a functional part thereof expressed from the vector includes an ER-retention signal. A preferred ER-retention signal is HDEL (SEQ ID NO: 1). The α -1,2-mannosidase coding sequence can be operable linked to a constitutive or inducible promoter, and a 3' termination sequence. The vectors can be integrative vectors or replicative vectors. Particularly preferred α -1,2-mannosidase expression vectors include pGAPZMFManHDEL, pGAPZMFManMycHDEL, pPICZBMFManMycHDEL, pGAPZmManHDEL, pGAPZmMycManHDEL, pPIC9mMycManHDEL and pGAPZmMycManHDEL.

Please amend the paragraph beginning on page 4, line 10 as follows:

In another preferred embodiment, the knock-in vectors of the present invention include a sequence coding for a glucosidase II or a functional part thereof and are capable of expressing the glucosidase II or the functional part in a methylotrophic yeast strain. A preferred nucleotide sequence is a nucleotide sequence encoding the glucosidase II of a fungal species, and more preferably, *Saccharomyces cerevisiae*. Preferably, the glucosidase II expression vector is engineered such that the glucosidase II or a functional part thereof expressed from the vector includes an ER-retention signal. A preferred ER-retention signal is HDEL (SEQ ID NO: 1). The glucosidase II coding sequence can be operable linked to a constitutive or inducible promoter, and a 3' termination sequence. The vectors can be integrative vectors or replicative vectors. Particularly preferred glucosidase II expression vectors include pGAPZAGLSII, pPICZAGLSII, pAOX2ZAGLSII, pYPTIZAGLSII, pGAPADEglsII, pPICADEglsII, pAOX2ADEglsII, pYPTIADEglsII, pGAPZAglsIIHDEL and pGAPADEglsIIHDEL.

Please amend the paragraph beginning on page 6, line 19 as follows:

Figure 1 depicts vectors carrying an HDEL (SEQ ID NO: 1)-tagged *Trichoderma reesei* α -1,2-mannosidase expression cassette and describes the way in which these vectors were constructed according to methods known in the art. Abbreviations used throughout construction schemes: 5' AOX1 or AOX1 P: *Pichia pastoris* AOX1 promoter sequence; Amp R: ampicillin resistance gene; ColE1: ColE1 origin of replication; 3'AOX1: 3' sequences of the *Pichia pastoris* AOX1 gene; HIS4: HIS4 gene of *Pichia pastoris*. AOX TT: transcription terminator sequence of the *Pichia pastoris* AOX1 gene; ORF: open reading frame; S: secretion signal; P TEF1: the promoter sequence of the *Saccharomyces cerevisiae* transcription elongation factor 1 gene; P EM7: synthetic constitutive prokaryotic promoter EM7; Zeocin: Zeocin resistance gene; CYC1 TT: 3' end of the *S. cerevisiae* CYC1 gene; GAP: promoter sequence of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene; PpURA3: *Pichia pastoris* URA3 gene. As can be seen in this figure, the *Trichoderma reesei* α -1,2-mannosidase was operably linked to the coding sequence for the *S. cerevisiae* α -mating factor secretion signal sequence and further operably linked at the 3' terminus of the coding sequence to the coding sequence for an HDEL (SEQ ID NO: 1) peptide. The whole fusion construct was operably linked to either the *P. pastoris* AOX1 promoter (in pPIC9MFManHDEL) or to the *P. pastoris* GAP promoter (in pGAPZMFManHDEL).

Please amend the paragraph beginning on page 7, line 9 as follows:

Figure 2 depicts vectors carrying an HDEL (SEQ ID NO: 1)-tagged *Mus musculus* α -1,2-mannosidase IB expression cassette and describes the way in which these vectors were constructed according to methods known in the art. As can be seen in this figure, the catalytic domain of the *Mus musculus* α -1,2-mannosidase IB was operably linked to the coding sequence

for the *S. cerevisiae* α -mating factor secretion signal sequence and further operably linked at the 3' terminus of the coding sequence to the coding sequence for an HDEL (SEQ ID NO: 1) peptide. The whole fusion construct was operably linked to either the *P. pastoris* AOX1 promoter (in pPIC9mManHDEL) or to the *P. pastoris* GAP promotor (in pGAPZmManHDEL). Furthermore, variants of the expression cassette were made in which the coding sequence for a cMyc epitope tag was inserted between the coding sequence for the *S. cerevisiae* α -Mating Factor secretion signal sequence and the coding sequence for the catalytic domain of the *Mus musculus* α -1,2-mannosidase IB. This expression cassette was also operably linked to either the *P. pastoris* AOX1 promoter (in pPIC9mMycManHDEL) or to the *P. pastoris* GAP promotor (in pGAPZmMycManHDEL).

Please amend the paragraph beginning on page 9, line 19 as follows:

Figure 7 depicts the processing of influenza haemagglutinin N-glycans by HDEL (SEQ ID NO: 1)-tagged *Trichoderma reesei* α -1,2-mannosidase and the HDEL (SEQ ID NO: 1)-tagged catalytic domain of murine α -1,2-mannosidase IB. The Man₅GlcNAc₂ reference oligosaccharide runs at scan 1850 in this analysis (not shown). Panel 1: malto-oligosaccharide size reference ladder. Panel 2: N-glycans derived from recombinant influenza haemagglutinin expressed in *Pichia pastoris*. The peak at scan 2250 corresponds to Man₉GlcNAc₂. Panel 3: N-glycans derived from recombinant haemagglutinin co-expressed in *Pichia pastoris* with *T. reesei* mannosidase-HDEL (under control of the GAP promotor). The peak at scan 1950 corresponds to Man₆GlcNAc₂. Panel 4: Same analytes as for panel 3, but after overnight treatment with 30 mU purified recombinant *T. reesei* α -1,2-mannosidase. Panel 5: N-glycans derived from recombinant haemagglutinin co-expressed in *Pichia pastoris* with mouse mannosidase IB-HDEL (under

control of the GAP promotor). Panel 6: same analytes as for panel 5, but after overnight treatment with 30 mU purified recombinant *T. reesei* α -1,2-mannosidase.

Please amend the paragraph beginning on page 16, line 13 as follows:

Multiple choices of ER retention signals are available to those skilled in the art, e.g., the first 21 amino acid residues of the *S. cerevisiae* ER protein MNS1 (Martinet et al. *Biotechnology Letters* 20: 1171-1177, 1998). A preferred ER retention signal for use in the present invention is peptide HDEL (SEQ ID NO: 1). The HDEL (SEQ ID NO: 1) peptide sequence, found in the C-terminus of a number of yeast proteins, acts as a retention/retrieval signal for the ER (Pelham *EMBO J.* 7: 913-918, 1988). Proteins with an HDEL (SEQ ID NO: 1) sequence are bound by a membrane-bound receptor (Erd2p) and then enter a retrograde transport pathway for return to the ER from the Golgi apparatus.

Please amend the paragraph beginning on page 17, line 18 as follows:

According to the present invention, the nucleotide sequence encoding a glucosidase II can derive from any species. Glucosidase II genes have been cloned from a number of mammalian species including rat, mouse, pig and human. The glucosidase II protein from these mammalian species consists of an alpha and a beta subunit. The alpha subunit is about 110 kDa and contains the catalytic activity of the enzyme, while the beta subunit has a C-terminal HDEL (SEQ ID NO: 1) ER-retention sequence and is believed to be important for the ER localization of the enzyme. The glucosidase II gene from *S. cerevisiae* has also been cloned (ORF YBR229c, located on chromosome II). This gene encodes a protein of about 110 kDa, which shows a high degree of homology to the mammalian alpha subunits.

Please amend the paragraph beginning on page 18, line 11 as follows:

In a preferred embodiment of the present invention, the glucosidase II protein is engineered to

include an ER retention signal such that the protein expressed in a methylotrophic yeast strain is targeted to the ER and retains therein for function. ER retention signals are as described hereinabove, e.g., the HDEL (SEQ ID NO: 1) peptide sequence.

Please amend the paragraph beginning on page 32, line 6 as follows:

The *Trichoderma reesei* α -1,2-mannosidase gene has been isolated and described by Maras et al. (*J. Biotechnol.* 77;255-263, 2000). The sequence of this gene is available at NCBI Genbank under Accession No. AF212153. A construction fragment was generated by PCR using the pPIC9MFmanase plasmid (same as pPP1MFmds1 described by Maras et al. (2000)) as the template and using the following oligonucleotide primers: 5'-GACTGGTTCCAATTGACAAGC-3' (SEQ ID NO:2) and 5'-AGTCTAGATTACAACCTCGTCGTGAGCAAGGTGGCCGCCCCG TCG-3' (SEQ ID NO:3). The resulting product contained the 3' end of the *Pichia pastoris* AOXI promoter, the prepro-signal sequence of the *S. cerevisiae* α -mating factor, the open reading frame of the *Trichoderma reesei* α -1,2-mannosidase cloned in frame with the signal sequence, the coding sequence for HDEL (SEQ ID NO:1), a stop codon and an *Xba* I restriction site. This fragment was digested with *Eco* RI and *Xba* I, removing the 5' sequences up to the mannosidase ORF, and then cloned into the vector pGAPZ α A (Invitrogen, Baarn, The Netherlands) which had been digested with *Eco* RI and *Xba* I, thus restoring the fusion with the *S. cerevisiae* α -mating factor signal sequence. The resulting plasmid was named pGAPZMFManHDEL and is graphically depicted in **Figure 1**. The ORF sequence of the MFManHDEL fusion in pGAPZMFManHDEL is set forth in SEQ ID NO: 14.

Please amend the paragraph beginning on page 33, line 4 as follows:

In order to introduce the coding sequence for a c-Myc tag between the catalytic domain and the

HDEL-signal (SEQ ID NO: 1), the 3' end of the ORF of *T. reesei* α -1,2-mannosidase was PCR-amplified using a sense primer 5'-CCATTGAGGACGCATGCCGCGCC-3' (SEQ ID NO: 4)

(containing an *Sph* I restriction site) and an antisense primer

GTATCTAGATTACAACCTCGTCGTGCAGATCCTCTTCTGAGATGAGTTTTTGTTCAGCA
AGGTGGCCGCCCCGTCGTGATGATGAA (SEQ ID NO: 5) (containing the coding sequences

of the c-Myc tag and the HDEL (SEQ ID NO: 1) signal, followed by a stop codon and an *Xba*

I restriction site). The resulting PCR product was digested with *Sph* I and *Xba* I, purified by

agarose gel electrophoresis and inserted into pGAPZMFManHDEL which had been cut with the

same restriction enzymes, resulting in plasmid pGAPZMFManMycHDEL. To put the ORF of

pGAPZMFManMycHDEL under the control of the inducible AOXI promoter, the entire ORF

was liberated from pGAPZMFManMycHDEL with *Bst* BI and *Xba* I, and cloned in pPICZB

(Invitrogen, Baarn, The Netherlands), resulting in pPICZBMFManMycHDEL.

Cloning of the mouse mannosidase IB catalytic domain with concomitant addition of the coding

sequence for a C-terminal HDEL-tag (SEQ ID NO: 1) was done by PCR on a mouse cDNA

library (mRNA isolated from the L929 cell line induced with cycloheximide and mouse Tumor

Necrosis Factor. Average insert length of the cDNA library was 2000 bp). The PCR

oligonucleotide primers used were: 5'AACTCGAGATGGACTCTTCAAAACACAAACGC3'

(SEQ ID NO: 6) and

5'TTGCGGCCGCTTACAACCTCGTCGTGTCGGACAGCAGGATTACCTGA3' (SEQ ID NO:

7). The product contained a 5' *Xho* I site and the coding sequence for C-terminal HDEL-site,

followed by a stop codon and a *Not* I site at the 3' end. The product was cloned in pGAPZ α A

via the *Xho* I /*Not* I sites in the PCR product and the vector, resulting in an in frame fusion of the

mouse mannosidase catalytic domain with the *S. cerevisiae* α -mating factor signal sequence. The

sequence of the entire open reading frame generated is set forth in SEQ ID NO: 15.

Please amend the paragraph beginning on page 36, line 21 as follows:

The results shown in **Figure 4** indicated that the great majority of the HDEL (SEQ ID NO: 1)-tagged protein was retained intracellularly, both when expressed from the strong constitutive GAP promoter and when expressed from the strong inducible AOXI promoter.

Please amend the paragraph beginning on page 36, line 27 as follows:

Isopycnic sucrose density gradient centrifugation -- To determine the localization of the HDEL (SEQ ID NO: 1)-tagged mannosidase, subcellular fractionation was carried out using cells expressing the mannosidase-Myc-HDEL from the strong constitutive GAP promoter.

Please amend the paragraph beginning on page 37, line 21 as follows:

The results illustrated almost exact cosedimentation of the MFManMycHDEL protein with the Protein Disulfide Isomerase marker protein (which is also targeted with a HDEL (SEQ ID NO: 1) signal sequence) (**Figure 5**). In the same assay, the HA-tagged OCH1 was distributed over the whole gradient, with the highest abundance in fractions having a density lower than that of the fractions containing the mannosidase and the PDI. This result indicated that the mannosidase was targeted to the expected location (the ER-Golgi boundary) by the addition of an HDEL (SEQ ID NO: 1) signal. In contrast, the mannosidase without HDEL (SEQ ID NO: 1), expressed from inducible alcohol oxidase I promoter (which was of comparable strength as the GAP promoter), was secreted at a high level of about 20 mg/l.

Please amend the paragraph beginning on page 45, line 1 as follows:

One of the Och1-inactivated clones was also further transformed with pGAPZMFManHDEL to produce "supertransformants". Both the Och1-inactivated clone and three supertransformants also expressing the ManHDEL were evaluated in cell wall glycan analysis as follows. Yeast cells

were grown in 10 ml YPD to an $OD_{600}=2$ and mannoproteins were prepared by autoclaving the yeast cells in 20 mM sodium citrate buffer pH7 for 90 min at 120 °C and recovery of the supernatant after centrifugation. Proteins were precipitated from this supernatant with 3 volumes of cold methanol. The protein preparation obtained in this way was used for N-glycan analysis using DSA-FACE as described by Callewaert et al. (2001) *Glycobiology* 11, 275-281. As shown in **Figure 10**, there was an increased amount of $Man_8GlcNAc_2$ glycan in the Och1-inactivated clone as compared to parent strain yGC4, indicative of a reduced activity of the Och1 enzyme. In all three supertransformants which also expressed the HDEL (SEQ ID NO: 1)-tagged α -1,2-mannosidase, the production of $Man_5GlcNAc_2$ was observed. Furthermore, upon digestion of the same glycan mixtures with 3 mU/ml purified recombinant *Trichoderma reesei* α -1,2-mannosidase, more $Man_5GlcNAc_2$ was formed in the strain transformed with pBLURA5'PpOCH1 than in the parent strain (**Figure 11**, compare panel 2 and 3).

Please amend the paragraph beginning on page 49, line 17 as follows:

Extracellular expression – PPY12-OH transformants of the construct pGAPZAGLSII(myhis6) (strains 12, 14 and 18) and transformants of the construct pGAPZAGLSII(myc)HDEL (strains H1, H2 and H3) were grown for 2 days on 2x10 ml BMDY medium. These 6 transformants earlier scored positive both on the genomic level (PCR on gDNA) and on the RNA level (Northern blot). The culture medium was collected by centrifugation and concentrated with Vivaspin columns to about 1 ml. Proteins from this concentrate were precipitated with TCA, resuspended in Laemmli buffer and loaded for SDS-PAGE analysis. Proteins were blotted to nitrocellulose membrane. The blot was incubated overnight with anti-myc Ab. The secondary Ab was linked to peroxidase. Using the Renaissance luminiscence detection kit (NEN) and a light sensitive film (Kodak), a strong band at about 110 kDa was observed for the transformants

12, 14 and 18, indicating that GLSII was expressed and secreted from these transformants. No signal was obtained for the transformants H1-3, which indicate that the HDEL (SEQ ID NO: 1) tag, which was added C-terminally to the GLSII ORF, resulted in an ER localization of the protein, preventing GLSII to be secreted into the growth medium.

Please amend the paragraph beginning on page 50, line 4 as follows:

Extracellular expression – PPY12-OH transformants of the construct pGAPZAGLSII(myhis6) (strains 12, 14 and 18) and transformants of the construct pGAPZAGLSII(myc)HDEL (strains H1, H2 and H3) were grown for 2 days on 2x10 ml BMDY medium. These 6 transformants earlier scored positive both on the genomic level (PCR on gDNA) and on the RNA level (Northern blot). The culture medium was collected by centrifugation and concentrated with Vivaspin columns to about 1 ml. Proteins from this concentrate were precipitated with TCA, resuspended in Laemmli buffer and loaded for SDS-PAGE analysis. Proteins were blotted to nitrocellulose membrane. The blot was incubated overnight with anti-myc Ab. The secondary Ab was linked to peroxidase. Using the Renaissance luminiscence detection kit (NEN) and a light sensitive film (Kodak), a strong band at about 110 kDa was observed for the transformants 12, 14 and 18, indicating that GLSII was expressed and secreted from these transformants. No signal was obtained for the transformants H1-3, which indicate that the HDEL (SEQ ID NO: 1) tag, which was added C-terminally to the GLSII ORF, resulted in an ER localization of the protein, preventing GLSII to be secreted into the growth medium.